FINAL REPORT

PROJECT NO. B-163

THE USE OF NITRATE INSTEAD OF AMMONIA AS THE INORGANIC NITRATE SOURCE IN BOD DILUTION WATER

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GRANT-IN-AID RG 6447(A)
FROM NATIONAL INSTITUTES OF HEALTH

MARCH 1960

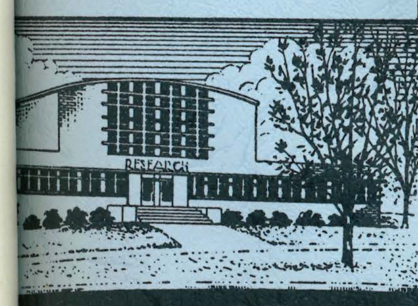

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TABLE OF CONTENTS

This report contains 21 pages.

LIST OF FIGURES

LIST OF TABLES

I. SUMMARY

Dilution water with sodium nitrate gives a more valid biochemical oxygen demand (BOD) with carbonaceous substrate with long periods of incubation than ammonia dilution water. Similar BOD values were obtained after 5 days incubation with nitrate and ammonia but even better agreement was obtained after 10 days. In a few tests the BOD value at 5 days with nitrate was lower than with ammonia due to a longer lag period with nitrate. In at least one test the 5-day BOD value was higher with nitrate due to a longer lag period in the test with ammonia. After 10 days any effect of lag period was overcome and results with ammonia and nitrate were in good agreement. When nitrogenous organic substrates such as glutamic acid were used, nitrate and ammonia gave the same value during the early period of incubation and this trend was followed to 20 days. With nitrogenous substrates the cumulative BOD curves using either nitrate or ammonia were very similar during the 20 days incubation.

With a domestic sewage substrate the amount of nitrification increased with increasing dilution of sample with both nitrate and ammonia dilution water. In this case, the BOD results were similar up to the tenth day, but due to heavy nitrification the 20-day BOD value with ammonia was considerably higher than with nitrate. In the study of pure carbonaceous material at low organic substrate concentration in the standard dilution test, the ammonia dilution water BOD value at 20 days was lower than at 10 days because there was considerably more nitrification in the seed blank than in the test bottle with organic material. This was due to the fact that in the absence of organic matter in the blank, essentially all of the ammonia was available for nitrification since there was essentially no heterotrophic assimilation.

II. INTRODUCTION

This project, involving a study of the use of nitrate instead of ammonia as the inorganic nitrogen source in BOD dilution water, was conceived from the results of an earlier study in which it was shown that the 5-day BOD results of the lower fatty acids were statistically comparable using either nitrogen source. More important, however, was the fact that in the latter part of the BOD incubation period, nitrification of the ammonia gave errors that were not involved when nitrate was used as the nitrogen source. In these tests, it was desired to obtain valid and reproducible carbonaceous BOD values over a longer period of time than the usual 5-day tests. It was desired to obtain ultimate carbonaceous oxygen demand values that are generally represented by a 20-day BOD value. Due to nitrification, the ultimate BOD value could not be obtained on lower fatty acids reliably when using the ammonia dilution water. It was shown that substitution of nitrate for the ammonia in the dilution water gave data that showed no increase between the time of maximum carbonaceous oxygen uptake and the completion of the 20-day incubation period.

The use of nitrate would be desirable for long-term BOD studies (20 days) when it is necessary to determine the ultimate BOD of normal biological food. The results of this research will guide the choice of the better nitrogen source to be used as standard dilution water for BOD tests as outlined in Standard Methods.

III. PROCEDURE

All tests were conducted according to the procedures outlined in the eleventh edition of Standard Methods for the Examination of Water, Sewage and Industrial Wastes. The Warburg respirometer was used with organic substrate concentrations of 100 mg/liter. In these tests, the inorganic nutrient concentration was increased. The nitrogen and phosphorous concentrations were increased to give an initial organic-substrate-to-nitrogen-to-phosphorous ratio of 100:5:1. In these tests five replicates were run for each reported value and readings were taken as necessary up to a period of 20 days.

The standard dilution method was used for lower substrate concentrations. In these tests, a modified dilution water was developed containing sodium nitrate nitrogen in the amount equivalent to that of ammonia nitrogen in the usual standard dilution water. In these tests three replicate bottles were run for each variable and the dissolved oxygen determinations generally were made after 3, 5, 7, 10 and 20 days of incubation.

IV. RESULTS

A. Sewage Analyses

The purpose of the initial tests with domestic sewage was to illustrate the problem of nitrification in the substrate most commonly analyzed within this field of interest and also to test the validity of our hypothesis concerning the availability of nitrate.

In Figure 1 and Table I was shown the results of standard dilution tests on three different concentrations of domestic sewage, namely, 2, 4 and 6 ml per bottle using both nitrate and ammonia dilution waters.

TABLE I

OXYGEN DEMAND OF THREE DIFFERENT CONCENTRATIONS OF DOMESTIC SEWAGE. NITRATE COMPARED WITH AMMONIA AS THE NITROGEN SOURCE IN BOD DILUTION WATER

Days	2 ML NH_3	2 ML NO_3	4 ML NH_3	4 ML NO_3	6 ML NH_3	6 ML NO_3
			(Parts Per Million)			
3	0.9	0.8	1.6	1.2	2.6	2.4
5	1.4	1.2	2.2	2.3	3.4	3.3
10	1.5	1.5	3.2	3.2	3.2	3.5
20	4.2	2.4	5.5	4.1	5.9	5.6

These data show that there was no nitrification in either set of blanks since there was no seed used in the blanks and that the difference between 20-day values with the nitrate and ammonia decreases with increasing sample volume per bottle, or with increasing amounts of nitrogenous and organic materical. It can be seen (Figure 1) that there is some nitrification even with the nitrate dilution water at the highest sewage

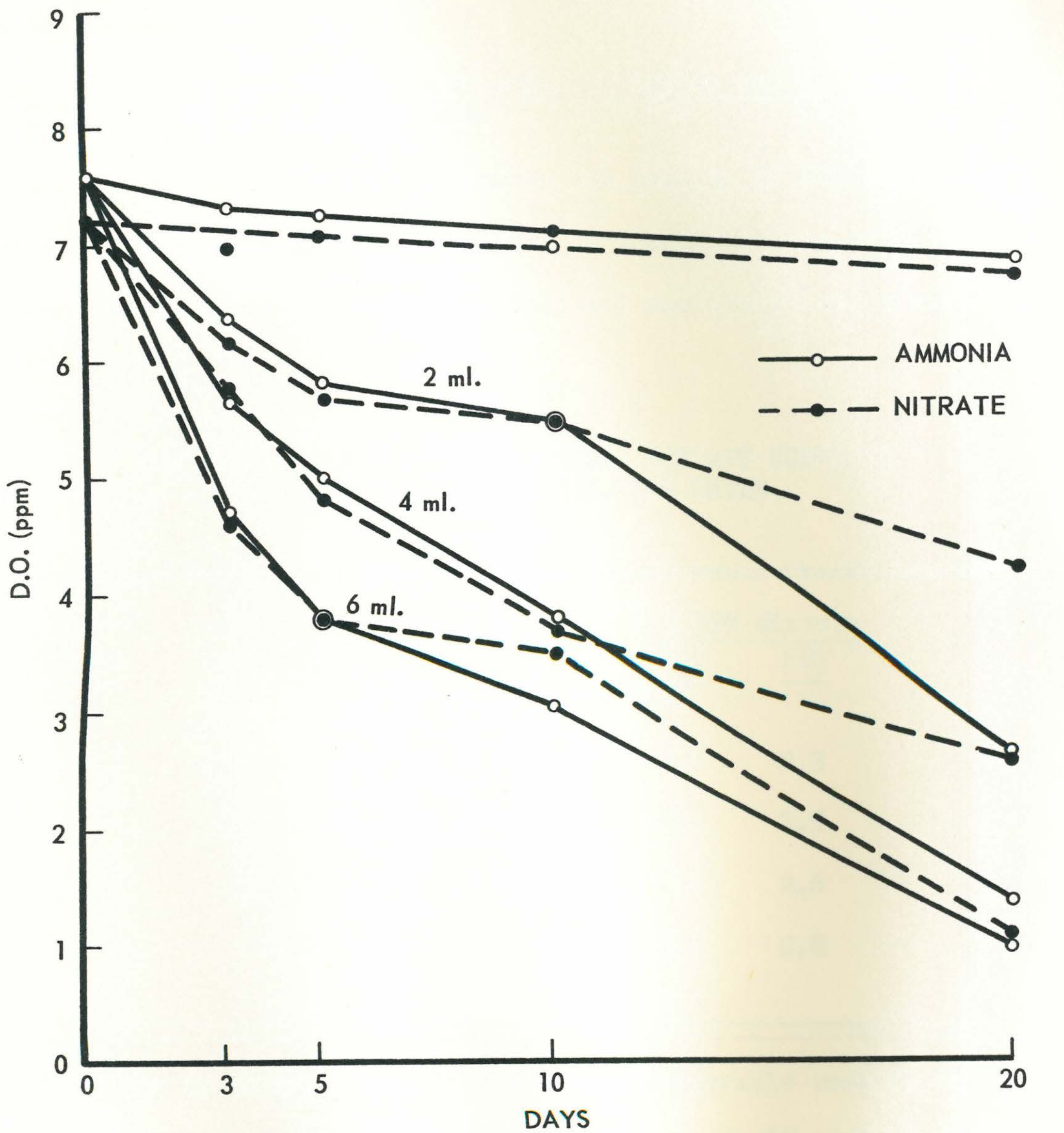

Figure 1. Oxygen Depletion in Bottles Using 2, 4, and 6 ml Domestic Sewage, with Nitrate and Ammonia Dilution Water.

concentration. The extent of nitrification is less than appears with the ammonia dilution water. The nitrification in the bottles with the nitrate dilution water is caused by the fact that some ammonia was introduced with the sewage substrate.

B. Effect of Nitrogen Source on Standard Dilution and Warburg BOD Values Using Pure Organic Compounds

In Figure 2 and Table II are shown the results of BOD measurements using a glutamic acid glucose substrate in concentrations of 3.0 and 6.0 ppm organic matter with both nitrate and ammonia dilution waters.

TABLE II

BOD OF GLUCOSE-GLUTAMIC MIXTURE. NITRATE COMPARED WITH AMMONIA IN THE DILUTION WATER

Days	3.0 PPM Mixture		6.0 PPM Mixture	
	NH_3	NO_3	NH_3	NO_3
	(Parts Per Million)			
2	1.1	1.1	2.1	2.3
3	1.2	1.2	2.4	2.5
5	1.0	1.1	2.4	2.6
7	1.2	1.2	2.6	2.8

The BOD values up to a period of 7 days show comparable results in each case. The seed blank curve indicates the sewage itself would have a 7-day BOD in the neighborhood of 200 to 350 ppm while the shape of the curve indicates that there was probably no nitrification in the seed up to that time. That comparable BOD values using nitrate and ammonia dilution water can be obtained throughout 20 days of incubation with a nitrogenous substrate is shown in Table III, which includes the BOD values with glutamic acid in addition to glucose, formate, acetate, citrate and

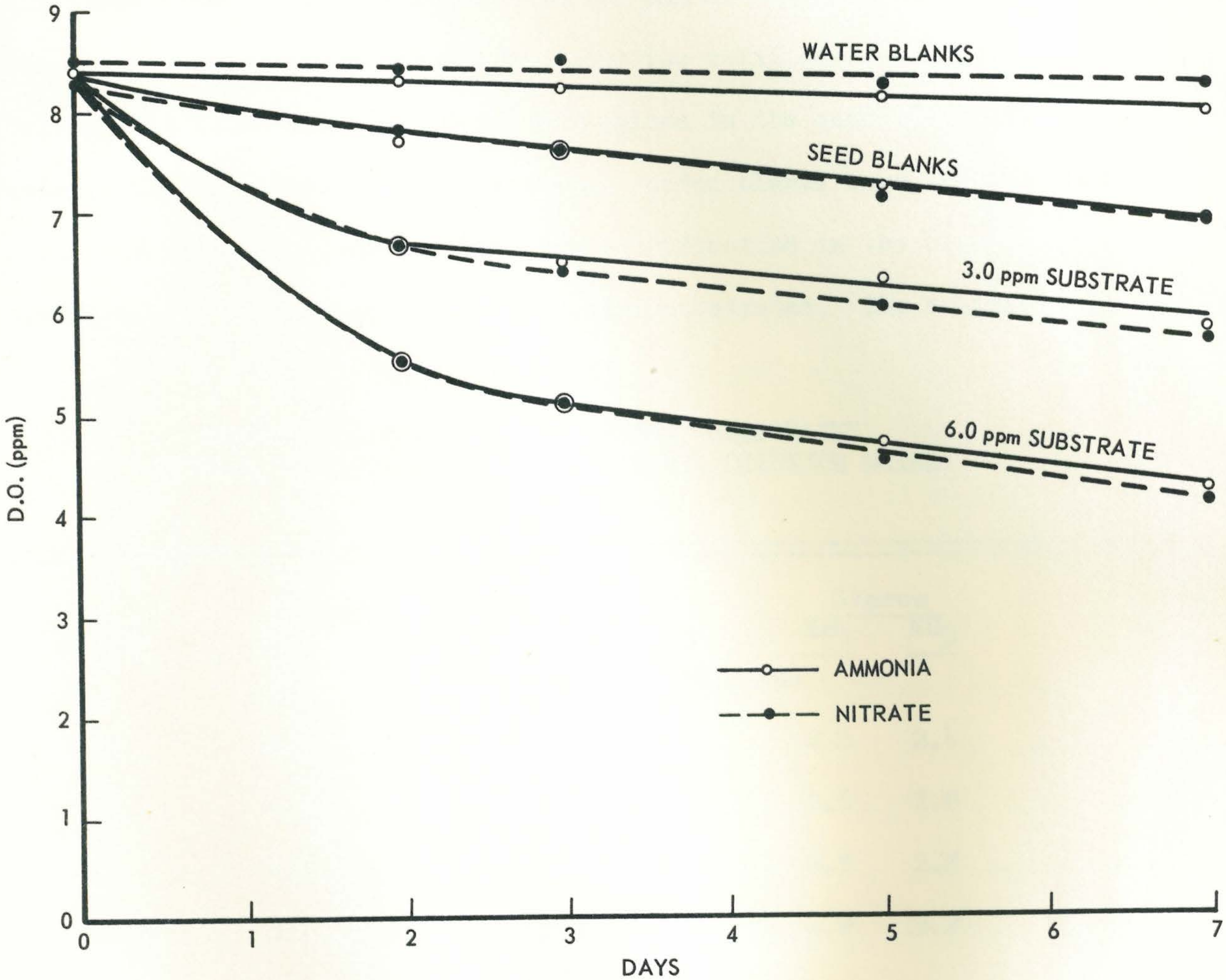

Figure 2. Deoxygenation of Glucose-Glutamic Acid Mixtures Using Nitrate and Ammonia Dilution Water.

starch. The data in Table III show that the 20-day BOD values with glutamic acid were comparable using both dilution waters. With glutamic acid it can be seen that the BOD values with the ammonia dilution water increased from 3.3 ppm at 10 days to 3.7 ppm at 20 days; however, with the other purely carbonaceous substrates the relative 10 and 20-day values were lower than the 10-day BOD values in the ammonia dilution water. This is caused by the fact that seeded blanks were used in these tests and there was considerably more nitrification in the blank than there was in the bottles with the organic substrates. The subtraction

TABLE III

OXYGEN DEMAND OF SEVERAL ORGANIC SUBSTRATES
NITRATE COMPARED WITH AMMONIA IN THE DILUTION WATER

	Glutamate		Citrate		Starch	
Days	NH_3	NO_3	NH_3	NO_3	NH_3	NO_3
			(Parts Per Million)			
3	2.3	2.5	3.2	3.2	2.2	2.4
5	2.8	3.3	3.6	3.5	2.6	2.8
10	3.3	3.3	4.2	3.5	2.9	2.8
20	3.7	4.0	3.5	3.0	1.9	3.2
	Glucose		Formate		Acetate	
	NH_3	NO_3	NH_3	NO_3	NH_3	NO_3
			(Parts Per Million)			
3	2.8	0	0.4	0.3	5.5	0.2
5	2.9	2.2	2.0	0.6	5.8	5.5
10	3.1	2.6	5.4	5.4	6.2	5.4
20	2.4	3.1	4.8	5.1	5.2	5.8

of the oxygen demand of the seed blank from the total oxygen demand in the substrate bottles resulted in the decreasing BOD. With the exception of formate and citrate substrates in the nitrate tests, the 20-day BOD values were slightly higher than the 10-day values. There seems to be a general tendency for the 20-day values with nitrate to approximate the 10-day values with ammonia dilution water and if no lag periods are involved, the 10 and 20-day nitrate dilution water values are comparable.

In order to study rates of oxygen uptake at higher organic matter concentrations, the Warburg respirometer was used with glucose. The results of the Warburg respirometer test with 100 ppm glucose are presented in Table IV and Figure 3. Five replicates of each variable were run with nitrate and ammonia dilution waters. The results showed that there was a longer lag period with the nitrate dilution water and that lower BOD values were obtained over the entire incubation period up to 22 days. The cause of these lower BOD results was attributed to the fact that the thermobarometer used in the test contained a high seed concentration with a mixture of nitrate and ammonia. The lower relative BOD was a result of subtracting the oxygen uptake in the thermobarometer caused by nitrification from the nitrate tests where no nitrification was occurring. For comparison with the respirometer results, a standard 5-day dilution test with both dilution waters was also prepared with a glucose substrate. The results in Table V show that minimum, maximum, and average BOD's with nitrate and ammonia were essentially identical and that the 5-day value using ammonia dilution water was essentially the same in each method.

TABLE IV

BOD OF 100 PPM GLUCOSE.
NITRATE COMPARED WITH AMMONIA IN THE DILUTION
WATERS WITH THE WARBURG TECHNIQUE

Days	NH_3 Min.	NH_3 Max.	NH_3 Avg.	NH_3 Dev.	NO_3 Min.	NO_3 Max.	NO_3 Avg.	NO_3 Dev.
	(Parts Per Million)				(Parts Per Million)			
1.3	0	2.2	1.0	±0.8	0	3.5	1.0	1.5
1.9	2.1	36	16	8.1	0	26	11	8.2
2.3	0	14	34	7.0	22	36	30	4.5
2.9	40	47	44	2.0	27	40	34	4.0
3.2	42	50	47	9.5	28	42	36	4.2
3.9	44	50	48	2.1	29	41	36	3.8
4.3	44	51	49	2.0	31	46	37	3.8
5.1	45	54	50	2.5	32	46	38	5.0
6.0	44	55	50	3.0	32	45	40	5.0
7.0	46	57	50	3.0	33	47	41	5.0
9.9	47	68	52	6.5	33	49	43	4.7
14.0	46	68	54	6.0	31	51	42	5.0
21.1	42	64	53	5.8	25	55	43	8.0

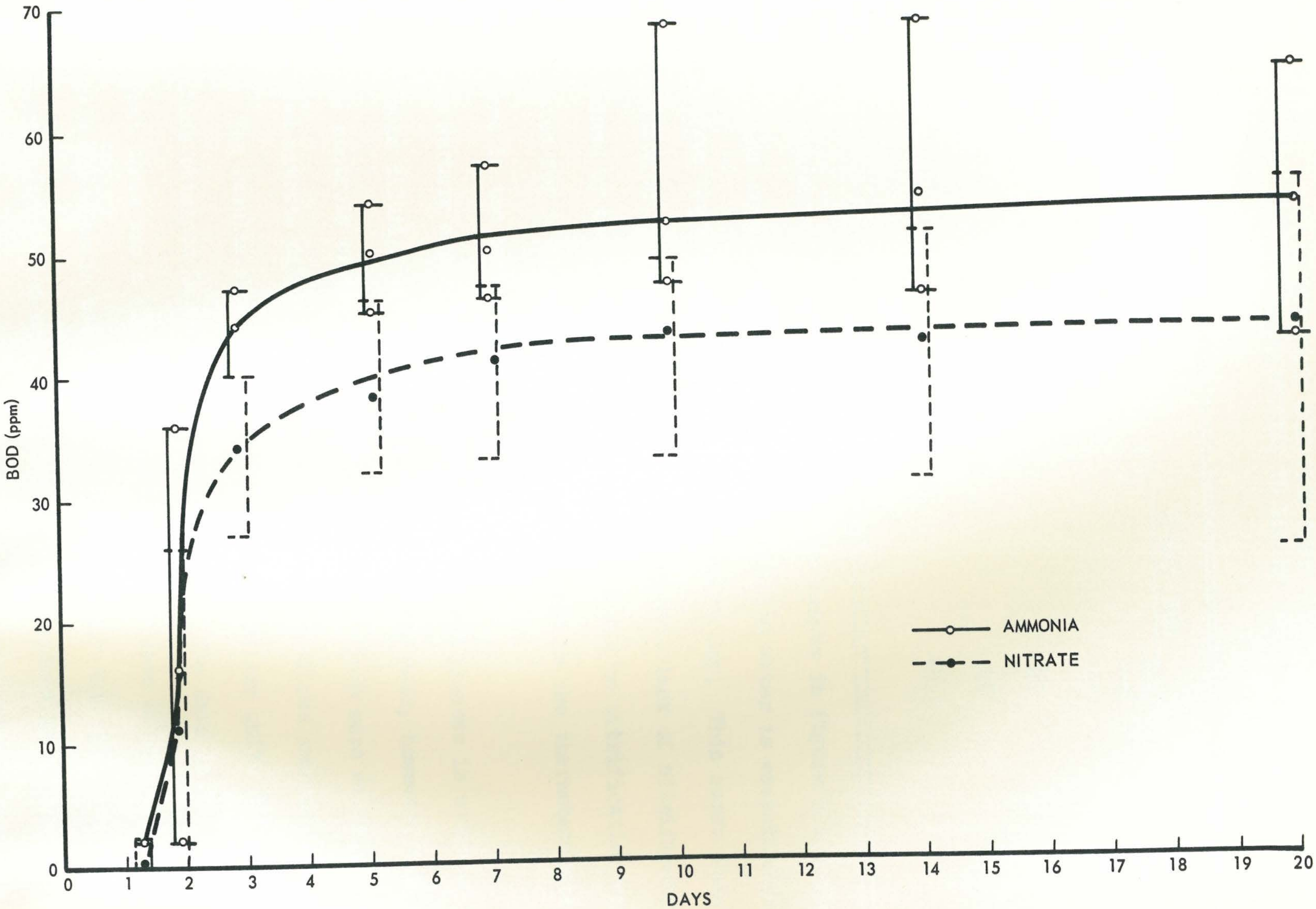

Figure 3. Oxygen Demand of 100 ppm Glucose Using Nitrate and Ammonia Dilution Water.

TABLE V

STANDARD DILUTION TEST
GLUCOSE EXTRAPOLATED TO 100 PPM USED

	5-Day BOD	
	NH_3	NO_3
Minimum, ppm	43	40
Maximum, ppm	49	49
Average, ppm	46	45
Deviation, ppm	±4.0	±5.0

The data shown in Table IV represent the curves in Figure 3 and also show that the oxygen demand using ammonia dilution water is essentially the same from the seventh day up to the twentieth day. This curve shows no indication of rapid nitrification. The apparent lack of nitrification is explained by the fact that the oxygen demand due to nitrification was compensated for in the subtraction of gas changes in the thermobarometer from those in the test flasks.

The Warburg experiments for comparing 100 ppm glucose in nitrate and ammonia dilution water were repeated. In this case, however, two types of controls or thermobarometers were used. Both were seeded, one contained nitrate dilution water and the other contained ammonia dilution water. The results shown in Figure 4 and Table VI are quite different from those obtained in the previous experiment. The data in Table VI show that there was a shorter lag period with nitrate dilution water than with the ammonia but that after the third day this difference from the shorter lag period was eliminated. The BOD values after the third day with the ammonia dilution water were considerably higher than those

TABLE VI

OXYGEN DEMAND OF 100 PPM GLUCOSE
NITRATE COMPARED WITH AMMONIA IN THE DILUTION WATER

Days	NH_3 Min.	NH_3 Max.	NH_3 Avg.	NH_3 Dev.	NO_3 Min.	NO_3 Max.	NO_3 Avg.	NO_3 Dev.
	(Parts Per Million)				(Parts Per Million)			
1.0	0.4	8.0	4.0	±2.4	1.0	8.0	6.0	2.2
1.9	1.0	12	3.0	4.8	6.0	18	10	4.8
2.9	43	52	48	3.2	29	49	39	4.8
3.7	46	52	49	2.4	38	49	43	3.3
4.7	52	55	53	1.1	38	45	42	2.3
6.0	53	61	57	2.1	38	47	43	4.0
7.0	56	65	59	2.1	39	49	46	4.7
11.0	54	76	62	6.5	42	51	47	4.8
14.0	56	81	66	6.0	40	49	46	4.9
17.0	59	87	70	6.0	46	56	48	4.7
22.0	70	39	78	6.0	38	51	46	5.0

with the nitrate. The difference between the BOD values with the ammonia and nitrate dilution waters kept increasing up to the twentieth day. The difference between the two values in the early part of the incubation is most likely caused by the contribution of oxygen from the nitrate ion as the nitrogen was assimilated with the glucose by the organisms. The larger differences in the later phase of the incubation are most likely caused by nitrification with the ammonia dilution water.

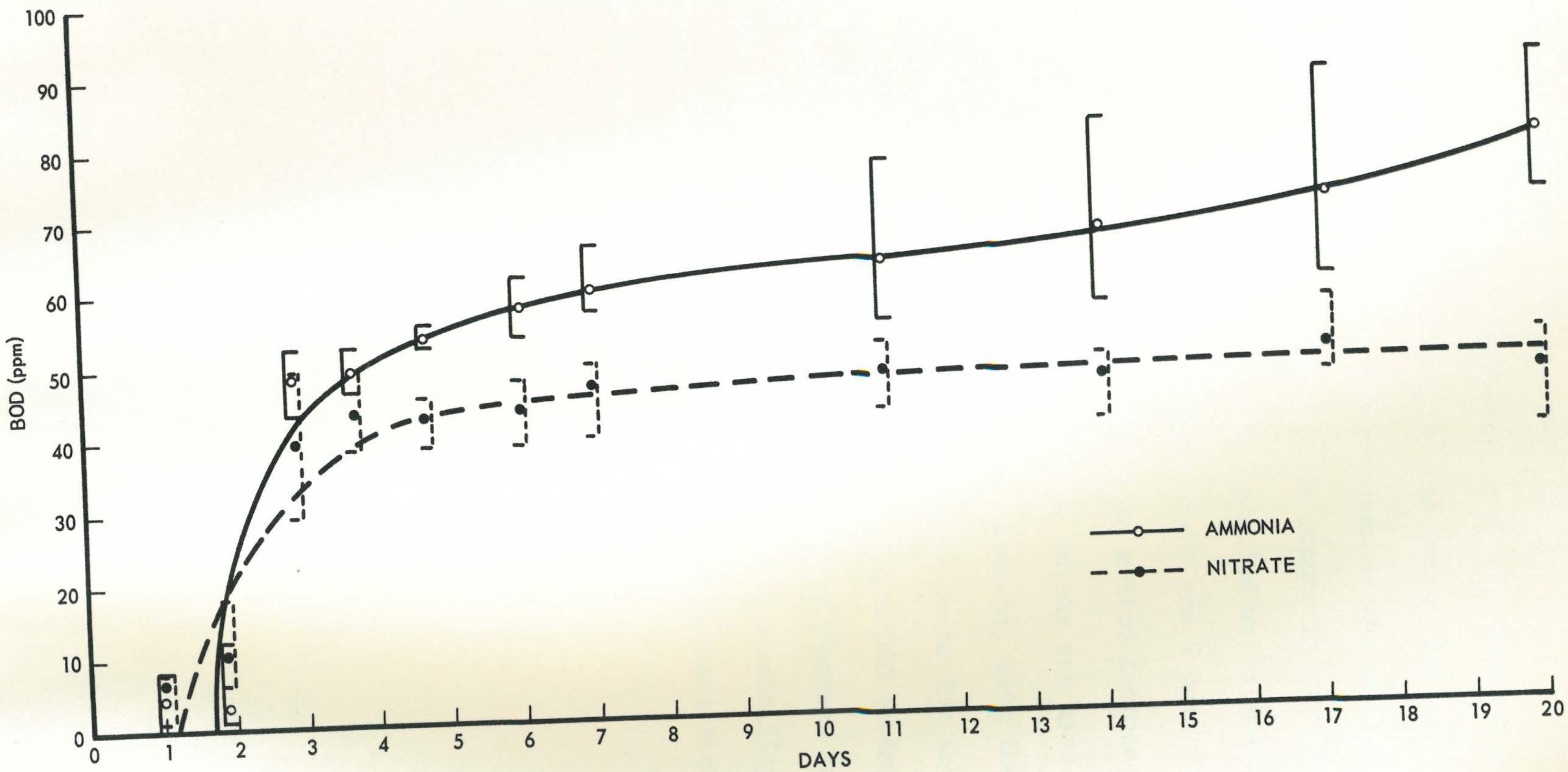

Figure 4. Oxygen Demand of 100 ppm Glucose Using Nitrate and Ammonia Dilution Water.

C. Investigation of Possible Toxicity of the Distilled Water Used

The BOD results obtained thus far with glucose are considerably lower than those obtained in previous experiments and from perviously published reports. An attempt was made to determine the reason for these lower values. It was considered possible that the lower oxygen demand value might be caused by either inorganic metal ions such as copper in the dilution water, or by monochloramine, which occurs in the distilled water used in this laboratory. Several tests which were run using 3 and 6 ppm glucose-glutamic acid mixtures in the standard dilution method included a series in which the distilled water from the building supply was used and another in which deionized distilled water was used. However, with these particular batches of distilled water, the qualitative tests for the presence of monochloramine were negative. The results of the BOD tests shown in Figure 5 and Table VII indicate that there was no difference in the BOD values with the distilled and deionized water. The possibility still exists, however, that monochloramine may have been present and may have produced the lower results of earlier experiments.

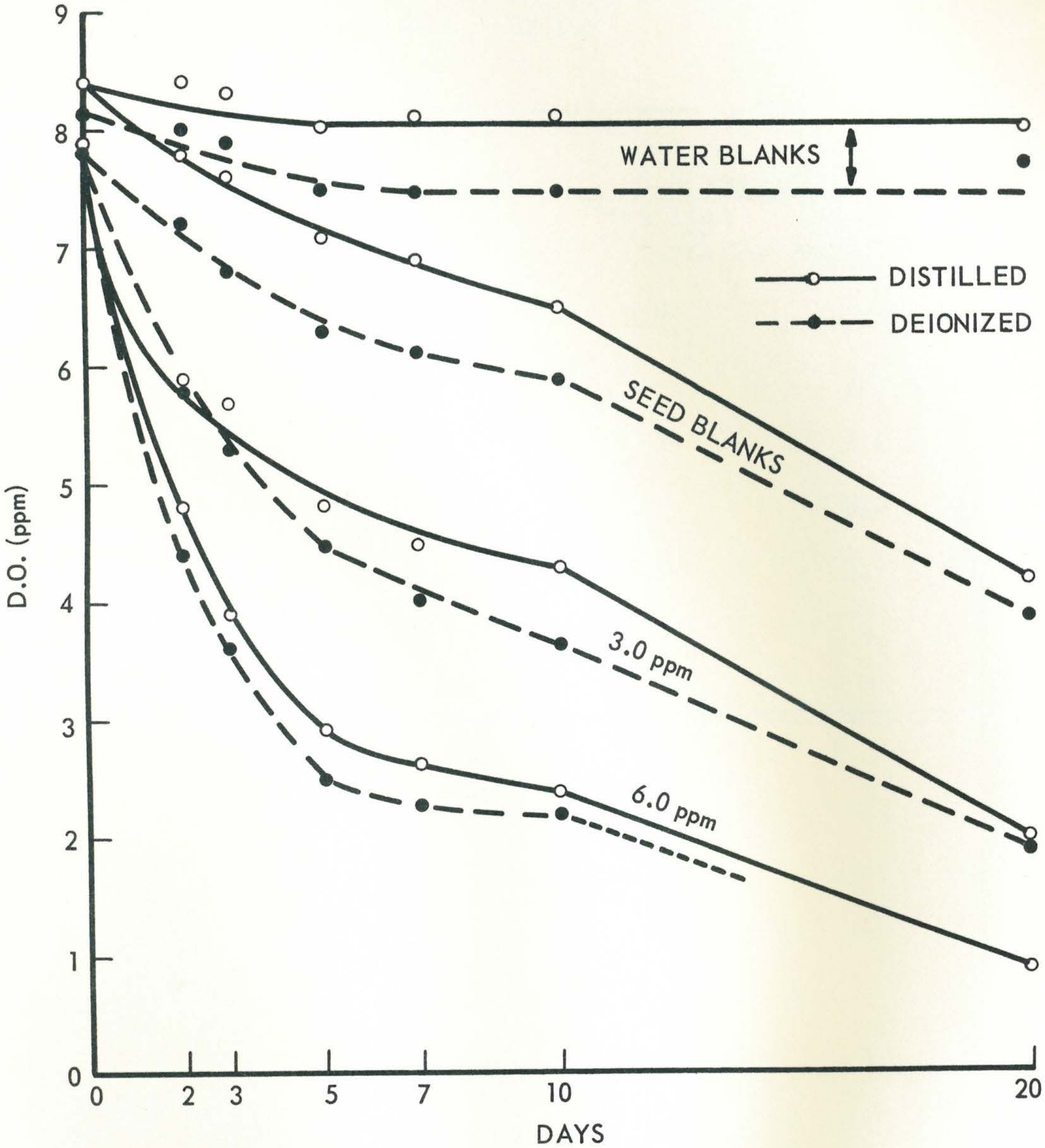

Figure 5. Deoxygenation of Glucose-Glutamic Acid Mixture Using Distilled and Deionized Standard Dilution Water.

TABLE II

COMPARISON OF DISTILLED AND DEIONIZED DILUTION WATER IN BOD TESTS WITH 3.0 AND 6.0 PPM GLUCOSE-GLUTAMIC ACID MIXTURES

Days	3.0 ppm Mixture		6.0 ppm Mixture	
	Distilled	Deionized	Distilled	Deionized
	(Parts Per Million)		(Parts Per Million)	
2	1.4	1.4	2.5	2.8
3	1.4	1.5	3.2	3.2
5	1.8	1.8	3.7	3.8
7	2.0	2.1	3.8	3.8
10	1.7	2.3	3.6	3.7
20	1.7	2.0	2.8	-

V. GENERAL DISCUSSION

Whether nitrification in the BOD test is a problem depends upon various circumstances. When determining the BOD of a non-nitrogenous substrate that has its own mixed seed, there is no necessity of seeding the blanks. With a long incubation period nitrification can be observed with ammonia dilution water because the total oxygen demand will be the sum of the total carbonaceous and nitrogenous BOD. In standard dilution tests on substrates that require artificial seeding with domestic sewage a different possibility exists. Since the blanks are seeded also, nitrification will occur in these blanks during a long incubation period. The nitrification in the blanks may exceed that in the sample bottles because some of the ammonia has been consumed in forming bacterial protoplasm. Therefore, when subtracting the oxygen demand of the blanks from the total oxygen demand in the sample bottles, an error will arise from the fact that the nitrification might be greater in the blanks. This dilemma is acknowledged in Standard Methods where it is recommended that the BOD of the sewage seed itself be determined in higher strengths in the bottles and then an appropriate correction on this basis be used for the calculation of the organic substrate BOD. If this procedure is not used, however, the greater amount of nitrification in the blanks than in the tests will give values that decrease in the later phases of the curve. The BOD will decrease with time. The fact that the highest amount of nitrification will occur with least amounts of organic substrate is evident from the data in our experiments with domestic sewage. The BOD values with three different sewage concentrations show that nitrification began earliest with the lowest sewage concentration and the oxygen demand also reached the greatest value at the lowest concentration. This increasing BOD with decreasing sewage concentration is the result of the decreasing demand of the heterotrophs for oxygen and ammonia. Consequently, the availability of these substances for the nitrifiers is greater.

It is common practice in Warburg determinations to use as a thermobarometer a suspension containing the dilution water and the seed material. In the calculation of the BOD values it is the custom to subtract the gaseous uptake in the thermobarometer from the gaseous uptake in the test flasks. However, a difference develops in the blanks between the Warburg procedure and the standard dilution procedure because there is a potassium hydroxide vial to trap carbon dioxide from the atmosphere in the Warburg test; this in turn reduces the carbon dioxide in the contents of the Warburg flasks. Thus in the seed blank thermobarometer, even though there are organisms and ammonia, no carbon dioxide is available to nitrifiers that are present and, therefore, little or no nitrification occurs. A lack (lower rate) of nitrification in the blanks while there is a slow production of nitrites in sample bottles, produces a curve with a slight inflexion from the Warburg BOD results with the test solutions containing ammonia. After 10 days incubation, with rapid nitrification (and slow carbonaceous oxidation) the apparent Warburg BOD values increase while the standard dilution BOD values decrease because nitrification in the dilution blanks proceeds more rapidly than in the sample bottles (there is higher DO in the dilution blanks along with available carbon dioxide).

Since there are many little understood factors or variables coming into play in the comparison of nitrate and ammonia dilution waters, the investigators feel that at this time it is not possible to make a broad conclusion based on the data obtained in this research. The investigators do feel, however, that under certain research circumstances (especially long term BOD studies) the use of nitrate dilution water would be highly desirable, but they hesitate at this time to recommend the wide use of nitrate dilution water in BOD studies or for inclusion in the next issue of Standard Methods.

The frequent occurrence of lag periods has been responsible for many of the problems involved in the interpretation of these research data. It is felt that before further work is performed directed toward the comparison of nitrate and ammonia, some work will have to be done toward reducing or eliminating lag periods in BOD studies. From a study of the literature on pure cultures of heterotrophic bacteria it has been pointed out that these bacteria have an absolute requirement for carbon dioxide and that carbon dioxide's partial pressure is inversely related to the length of the lag period. It is believed by us that the carbon dioxide concentration might possibly be a factor affecting lag periods with the mixed cultures of heterotrophs and autotrophs involved in these BOD studies. Consequently, the investigators have submitted an additional research proposal to study the possible effect of adding carbon dioxide to shorten lag periods.

VI. CONCLUSIONS

(1) The use of nitrate instead of ammonia in BOD dilution water can, in some cases, eliminate the errors in nitrification in long term carbonaceous substrate BOD studies. On the other hand, the oxygen from the nitrate can be a factor in lowering the observed BOD of a substrate by the equivalent amount of nitrate nitrogen which is consumed.

(2) Because so many chance and planned factors enter into a decision concerning the desirability of substituting nitrate for ammonia (character of substrate, length of incubation, number of nitrifying organisms in the seed), nitrate is not recommended for inclusion as a mineral supplement in the dilution water as given in Standard Methods. It can be used to advantage for many research problems.

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